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Antiviral activity of lambda-carrageenan prepared from red seaweed (Gigartina skottsbergii) against BoHV-1 and SuHV-1

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ABSTRACT

The antiviral effect of polysaccharides has been known for many years. Carrageenans are considered a good alternative for the prevention of a wide range of diseases, mainly caused by enveloped viruses. The advantages lie on their high availability, low cost and low induction of resistance. The aim of this study was to evaluate the sensitivity of two viral pathogens of veterinary interest to the presence of lambda-carrageenan. This is the first report of a lambda-carrageenan having antiviral activity against animal viruses belonging to the *Alphaherpesvirinae* subfamily, BoHV-1 (bovine herpesvirus type 1) strain Cooper and SuHV-1 (suid herpesvirus type 1) strain Bartha. Lambda-carrageenan was able to reduce infectivity of both viruses with a more pronounced effect against BoHV-1. These results proved, as previously shown for human herpes virus type 1, that these compounds could be used as potential antiviral agents in the veterinary field.

Carrageenans are sulfated polysaccharides (SPs) synthesized in great quantities by certain red algae and have a linear structure of alternating 3-β-D-galactopyranose and 4-α-D-galactopyranose or 4-3,6-anhydrogalactopyranose residues, forming the disaccharide repeated units. The presence or absence of 3,6-anhydrogalactopyranose and the proportion and distribution of the sulfate groups differentiate the various types of carrageenans : *kappa/iota*, *mu/nu* and *lambda* carrageenans (Knutsen et al., 1994; McCandless and Craigie, 1979).

Lambda-carrageenan is extracted from tetrasporic plants of the red seaweed *Gigartina skottsbergii* (Gigartinaceae, Rhodophyta), abundant in Patagonian areas. Lambda-carrageenan has a very high sulfate content and only trace amounts of 3,6-anhydrogalactose (molar ratio, galactose:3,6-anhidrogalactose:sulfate, 1.00:0.06:1.77) (Carlucci et al., 1997).

It has been demonstrated that SPs have antiviral activity against human herpesvirus type 1 and type 2 (HSV-1, HSV-2), and human cytomegalovirus (CMV) (Carlucci et al., 1999; Damonte et al., 2004; Gonzalez et al., 1987; Matsuhiro et al., 2005; Pujol et al., 2006).

It has been suggested that the antiviral activity of lambda-carrageenan is due to its ability to bind to the viral envelope glycoproteins and prevent virus binding to cell surface receptors (Carlucci et al., 1997).

The purpose of this work was to evaluate the antiviral effect of lambda-carrageenan against two animal herpesviruses, bovine herpesvirus type 1 (BoHV-1) and suid herpesvirus type 1 (SuHV-1).

BoHV-1 is a worldwide disseminated pathogen displaying significant differences in regional incidence and prevalence in regards to geographical positions and breeding management of different regions (Ackermann and Engels, 2006; Muylkens et al., 2007). Similar to BoHV-1, SuHV-1 outbreaks occur in swine populations worldwide, resulting in substantial economic losses for affected countries (Pomeranz et al., 2005).

BoHV-1 can produce rhinotracheitis, clinically known as “red nose”. It may also produce conjunctivitis and sporadic abortions with acute necrotizing endometritis. BoHV-1 may also play a role in enzootic pneumonia (Jones, 2003; Schwyzer and Ackermann, 1996). In pigs, SuHV-1 is responsible for causing Aujesky’s disease. Focal necrosis can be observed in tissues especially in piglets, being highly lethal in this age group (McGavin and Zachary, 2007). This virus can also affect species such as sheep, cattle, dogs and cats (Mettenleiter, 1996).

BoHV-1 strain Cooper and SuHV-1 strain Bartha were propagated in MDBK (Madin-Darby Bovine Kidney) cells for viral stocks

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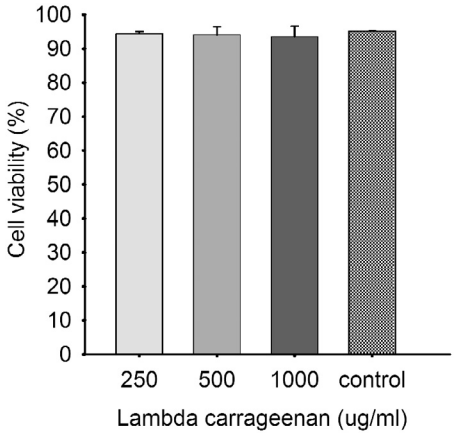

Fig. 1. Cytotoxicity determination of the lambda-carrageenan. Survival percentage of MBDK cells treated with different amounts of lambda-carrageenan and untreated control (100%).

using Eagle's minimum essential medium (MEM) (Gibco, Carlsbad, CA, USA) supplemented with 2% bovine fetal serum (BFS).

MDBK cells were grown in MEM supplemented with 5% BFS and 50 μg/ml gentamicin. For maintenance medium serum was reduced to 1.5%. The viral titer was determined by PFU (Plaque Forming Unit)/ml (Dulbecco, 1952).

Lambda-carrageenan was obtained and purified as described before (Carlucci et al., 1999). Briefly, carrageenans were extracted with water at room temperature and fractionated with KCl. Lambda carrageenan was precipitated from the fractionation of its tetrasporic form with KCl (0.60–0.70 M).

For the cytotoxicity determination, confluent monolayers of MDBK cells were treated with increasing amounts of lambda-carrageenan (250, 500, and 1000 μg/ml) in MEM. Each assay was performed in duplicate, analyzed 48 h later by treatment with trypsin and viability was determined counting cells by the Trypan blue method (Strober, 2001). A control was performed with cells treated identically but without the presence of the compound. Viability studies showed that lambda-carrageenan was not significantly cytotoxic to MDBK at the concentrations tested. Moreover, no cytotoxic effect was observed even when the concentration was increased to 1000 μg/ml (Fig. 1).

To evaluate the antiviral activity of lambda-carrageenan, a plaque reduction assay (PRA) was performed. Confluent monolayers of MDBK cells were grown in 24 well plates for 24 h. Cells were infected with 65 PFU of BoHV-1 or 70 PFU of SuHV-1, simultaneously with different concentrations of the polysaccharide in a total volume of 200 μl. For SuHV-1, lambda-carrageenan concentrations used were 0, 2, 5, 10, 15, and 25 μg/ml while for BoHV-1 0, 0.5, 0.75, 1 and 1.25 μg/ml were used. The range of concentrations was determined previously by pilot experiments. After 1 h of incubation at 37 °C, inocula were discarded and monolayers were covered with MEM with 1.5% methylcellulose without carrageenan. Incubation at 37 °C with 5% of CO_2 was continued for 48 h after which cells were fixed at –20 °C with methanol for 20 min and stained with crystal violet. PFU/ml titers were calculated and compared with controls without lambda-carrageenan treatment. For both viruses, two independent experiments, each with four determinations, were performed. The standard error of the mean (SEM) was calculated and antiviral activity was defined as the concentration required to reduce viral cytopathic effect to 50% (inhibitory concentration 50%, IC_{50}).

A negative control consisting of a viral suspension without carrageenan, and titrated by the same method, was included in every assay.

To determine whether the antiviral action of lambda carrageenan had an effect during BoHV-1 and SuHV-1 infection cycle, a yield reduction assay was performed. Confluent monolayers as in PRA were used. Plates were infected by adsorption of BoHV-1 or SuHV-1 (without carrageenan) at a multiplicity of infection (MOI) of 0.1 PFU per cell for 1 h at 37 °C. Cells were washed with warm medium and the same concentrations of lambda-carrageenan used in PRA were prepared in MEM and added immediately after virus adsorption. For SuHV-1 and BoHV-1 additional concentrations of carrageenan at 50–75 μg/ml and 1.5 μg/ml were tested respectively. At 48 h after virus inoculation, cells in the culture medium were lysed by freezing and thawing twice, and supernatant consisting of culture medium and cell lysate was obtained by centrifugation at 400 g for 15 min at 4 °C. Virus titer was determined by PFU assay in MDBK cells as described above in triplicate. The ability of the compound to inhibit virus production in mammalian cell culture was defined as the carrageenan concentration required for a 50% reduction on the progeny virus titer (yield concentration 50%, YC_{50}).

Finally, a virucidal assay was performed to study the possibility of an inactivating effect of the carrageenan against both viruses. Virucidal activity was calculated by performing a pre incubation of virus stocks with different concentrations of lambda-carrageenan during 90 min at 37 °C. Concentrations used for SuHV-1 were 0, 2, 5, 10, 15, 25, and 50 μg/ml while for BoHV-1 were 0.0, 0.5, 0.75, 1.0, 1.25, 1.50 and 2 μg/ml. Following this incubation step, the virus-compound mixture was first diluted to 1000-fold and 10 folded dilutions were used to infect confluent monolayers of MDBK cells. Cultures were incubated for 1 h, and then the medium was discarded. Finally, cultures were covered with MEM with 1.5% methylcellulose. Incubation at 37 °C with 5% of CO_2 was continued for 48 h after which cells were treated as for the PRA assay and PFU/ml titers were calculated as described before. Virucidal activity was defined as the concentration required to inactivate virions to 50% (virucidal concentration 50%, VC_{50}).

The lambda-carrageenan antiviral activity, evaluated through a PRA, was observed for both viruses. For BoHV-1, the calculated IC_{50} was 0.52 ± 0.01 μg/ml, while for SuHV-1 was 10.42 ± 0.88 μg/ml (Fig. 2 and Table 1). These results suggested that the lambda-carrageenan has antiviral activity against the strains tested (Fig. 2). SuHV-1 was 20-fold less sensitive to the antiviral effect than BoHV-1.

The effect of the presence of carrageenan on the yield of infectious virus is shown in Fig. 2. The data indicate that viral titers were reduced in presence of increasing concentrations of carrageenan. The 50% inhibitory concentration of yield (YC_{50}) was 1.37 ± 0.13 μg/ml for BoHV-1 and 73.54 ± 2.07 μg/ml for SuHV-1 (Fig. 2 and Table 1). These results were in agreement with those observed in the PRA suggesting a higher sensitivity of BoHV-1 to this antiviral compound.

Virucidal activity of SPs is believed to be caused by the formation of a stable virion-SP complex where binding is not reversible and hence the sites on the viral envelope required for virus attachment to host cells are occupied by the SP (Damonte et al., 2004; Harden et al., 2009). Fifty percent inhibitory concentration of virucidal assay (VC_{50}) was 0.96 ± 0.08 μg/ml for BoHV-1 and 31.10 ± 2.28 μg/ml for SuHV-1 (Fig. 2 and Table 1).

In particular, lambda-carrageenan was reported as an inactivating agent against herpes simplex virus whereas other structural types of carrageenans and SPs in general usually lack these inactivating properties (Talarico and Damonte, 2007). Our experiments show slightly higher carrageenan concentrations to achieve the VC_{50} values compared with those for IC_{50}. These differences however would not support the conclusion that the inactivating effect of the carrageenan is only due to a blockage in the binding of virions to the cell membrane, as observed for other viruses (Talarico and Damonte, 2007).

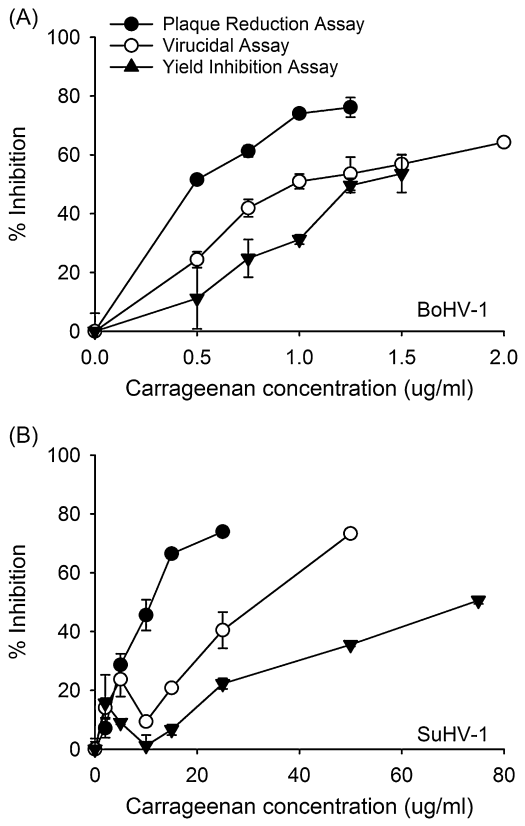

Fig. 2. The effect of the polysaccharide on BoHV-1 and SuHV-1 infection monitored by plaque assay in MDBK cell cultures. CPE inhibition percentage of untreated control (100%) of BoHV-1 (A) and SuHV-1 (B) infection in presence of different amounts of lambda-carrageenan for PRA (●),virucidal assay (○) and inhibition yield assay (▲). Values are indicated with its respective standard error mean (SEM).

Interestingly, differences in reactivity for both viruses as seen in this work, have been previously reported with other antiviral compound. For example, a stronger antiviral activity of heparin was observed against MDBK cells infected with a recombinant BoHV-1 strain expressing the SuHV-1 glycoprotein III (PrV gIII/gpC) while it was less active against a wild type BoHV-1 infection (Liang et al., 1993). As suggested by the authors, this could be due to differences in the interaction produced between the compounds and potential binding subregions of the viral glycoprotein C of both viruses.

It has been demonstrated that carrageenans are effective against other members of the herpesvirus family (Baba et al., 1988). The activity of a commercial lambda carrageenan with similar sulfate substitution pattern against HSV-1 and HSV-2 has been previously monitored on PRK cells (Primary rabbit kidney cells), being the IC_{50} for these agents 1.6 and 1.5 μg/ml respectively (Baba et al., 1988). Activity against CMV in HEL cells was also tested (Human erythroleukemia cell line) with an IC_{50} of 0.3 μg/ml reported (Baba et al., 1988).

Table 1
Fifty percent inhibition, virucidal and yield concentration of lambda-carrageenan on BoHV-1 and SuHV-1 infection.

	BoHV-1	SuHV-1
IC_{50}	0.52 ± 0.01SEM	10.42 ± 0.88SEM
VC_{50}	0.96 ± 0.08SEM	31.10 ± 2.28SEM
YC_{50}	1.37 ± 0.13SEM	73.54 ± 2.07SEM

Lambda-carrageenan showed no cytotoxicity on MDBK cells when its concentration was increased to 1000 μg/ml.

In summary, our results suggest that lambda-carrageenan may have applications in the veterinary field. Furthermore, the possibility of obtaining these natural compounds in large amounts from algae by inexpensive methods adds an interesting feature to its potential use. However, further studies should be performed to prove that lambda-carrageenan is a suitable alternative as therapeutic agent in-vivo against diseases caused by SuHV-1 and BoHV-1.

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